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PRODUCTION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES RECOGNIZING THE INTRACYTOPLASMIC THIRD LOOP OF THE 5-HYDROXYTRYPTAMINE_{1A} RECEPTOR

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Abstract—The portion of the complementary DNA encoding the third intracellular loop of the rat 5-hydroxytryptamine_{1A} (serotonin) receptor was subcloned into the vector pGEX-KG and expressed in *Escherichia coli* as a fusion protein coupled with the glutathione *S*-transferase of *Schistosoma japonicum*. The fusion protein was purified on a glutathione-agarose affinity column and used to immunize rabbits for the production of polyclonal anti-5-hydroxytryptamine_{1A} receptor antibodies. Enzyme-linked immunosorbent assay revealed that antibodies were produced as early as one month after the first injection of the fusion protein, and immune response plateaued at a maximum after the third (monthly) booster injection. These antibodies only marginally affected the specific binding of [³H]8-hydroxy-2-(di-*n*-propylamino) tetralin to solubilized and membrane bound 5-hydroxytryptamine_{1A} receptors, and did not interfere with serotonin-induced inhibition of forskolin-stimulated adenylate cyclase negatively coupled to 5-hydroxytryptamine_{1A} receptors in rat hippocampal membranes. However, antibodies were able to immunoprecipitate 5-hydroxytryptamine_{1A} receptor binding sites solubilized from rat hippocampal membranes. The distribution of immunautoradiographic labelling and immunohistochemical staining of rat brain sections exposed to the antibodies raised against the fusion protein superimposed to that of 5-hydroxytryptamine_{1A} receptor binding sites labelled by specific radioligands, with marked enrichment in the limbic areas (dentate gyrus and CA1 area in the hippocampus, lateral septum, entorhinal cortex) and the anterior raphe nuclei.

The differential cellular location of immunoreactivity within the hippocampus (where dendritic fields but not pyramidal cell somas were immunostained) and the median raphe nucleus (where the plasmic membrane of somas was strongly immunoreactive) suggests that the addressing of 5-hydroxytryptamine_{1A} receptors might differ from one neuronal cell type to another.

Among the multiple receptors for serotonin (5-hydroxytryptamine, 5-HT), the 5-HT_{1A} type has been extensively studied thanks to the development

of selective radioligands (see Ref. 18 for a review) and because partial agonists acting at this particular receptor have anxiolytic and antidepressant properties.^{25,45} Cloning and sequencing of the 5-HT_{1A} receptor indicated that it belongs to the superfamily of receptors coupled to guanyl nucleotide binding proteins (G proteins), with typical features such as seven transmembrane spanning domains, a glycosylated extracellular N-terminal domain and a short intracellular C-terminal domain.^{2,12,23}

In contrast to the transmembrane domains which exhibit a high degree of homology among the various G-protein coupled receptors, the third intracellular loop (i3) has a rather unique amino acid sequence which led to the use of synthetic peptides corresponding to parts of i3 as antigens for the possible development of anti-5-HT_{1A} receptor antibodies.^{9,12,23} Using this strategy, polyclonal antibodies recognizing the 5-HT_{1A} receptor from the rat and the human were produced, allowing the immunohistochemical

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Abbreviations: BSA, bovine serum albumin; cDNA, complementary DNA; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulphonate; DAB, 3,3'-diaminobenzidine tetrachloride; e2, second extracellular loop; EDTA, ethylenediaminetetra-acetate; ELISA, enzyme-linked immunosorbent assay; G protein, guanyl nucleotide binding protein; GST, glutathione *S*-transferase; GTP, guanosine-5'-triphosphate; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{1A}-i3/GST, fusion protein; i3, third intracellular loop; IPTG, isopropylthiogalactoside; LB-amp, Luria Broth-ampicillin; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline-0.1% Tween-20; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; (+)WAY 100135, (+)-*N*-1-butyl-3,4-(2-methoxyphenyl)-piperazine-1-yl-2-phenyl-propanamide.

labelling of material not only in the CNS^{9,12,29} but also in the kidney,³⁴ where, however, neither 5-HT_{1A} receptor specific binding sites (Riad *et al.*, unpublished observation) nor 5-HT_{1A} receptor mRNA³⁴ could be detected, at least in the rat. In addition, polyclonal antibodies were also raised against a synthetic peptide corresponding to the second extracellular loop (e2) of the 5-HT_{1A} receptor sequence,⁴ but the immunolabelling obtained with these antibodies was markedly different from that with the anti-i3 antibodies. In particular, glial cells exhibited immunoreactive 5-HT_{1A} receptor-like material labelled by the anti-e2 antibodies,³¹ whereas only neurons could be labelled by anti-i3 antibodies.¹⁹

Because of these discrepancies, we attempted to use a strategy other than making synthetic peptides as antigens for the production of specific anti-5-HT_{1A} receptor antibodies. A fusion protein consisting of the full i3 loop of the rat 5-HT_{1A} receptor coupled to glutathione S-transferase (5-HT_{1A}-i3 GST) was made in bacteria transfected with the constructed plasmid, purified on a glutathione-affinity column and used as an antigen in rabbits. The present paper describes the characteristics of these antibodies as assessed by enzyme-linked immunosorbent and immunoprecipitation assays, and immunautoradiographic and immunohistochemical labelling of rat brain sections.

EXPERIMENTAL PROCEDURES

Construction of the recombinant expression plasmid pGEN-KG

A portion of the rat gene corresponding to the i3 domain of the 5-HT_{1A} receptor (see Fig. 1A) was amplified (from nucleotides 762 to 1167—see Ref. 2) using polymerase chain reaction (PCR) and the following primers: AGGATCCGAATTCCTCTACGGGCGCATCTTCAGA (5' primer) and AGGATCCCTCGAGTCAGCCAGAGTCTTCACCGTCTT (3' primer). PCR amplification was performed using 30 cycles at 94 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min. The amplified material was purified using Sephadex beads (Pharmacia), restricted with Bam HI, and subcloned into Bam HI restricted, dephosphorylated, pSK⁺ bluescript plasmid (Stratagene, CA). A clone (5-HT_{1A}i3 pSK⁺ bluescript) in the proper orientation was selected and completely sequenced using the double-stranded dideoxy chain termination method of Sanger (Sequenase, USB Corp.). No differences were detected between the amplified segment and the sequence reported by Albert *et al.*² In order to insert the 5-HT_{1A}i3 loop in frame with the glutathione S-transferase (GST; EC 2.5.1.18, from *Schistosoma japonicum*), the 5-HT_{1A}i3 pSK⁺ bluescript was restricted with Xho I and the insert was purified on a 1.5% agarose gel to be subcloned into Xho I restricted, dephosphorylated, pGEN-KG plasmid.¹³ A pGEN-KG plasmid bearing the insert was chosen (pGEN-5-HT_{1A}i3 GST) and used for all subsequent studies.

Induction and purification of the 5-hydroxytryptamine_{1A}-i3 glutathione S-transferase fusion protein in Escherichia coli

A culture of XL-1 blue bacteria (Stratagene, CA), transformed with the plasmid pGEN-5-HT_{1A}i3 GST, was grown overnight, in 200 ml of Luria Broth containing 100 µg/ml ampicillin (LB-amp), at 37 °C, with vigorous agitation. Saturated cultures were then diluted 10-fold in

LB-amp and further incubated for 2 h at 37 °C. Isopropyl thiogalactoside (IPTG, 0.1 mM) was added and agitation at 37 °C was continued for another 1 h. The bacterial pellet was harvested by centrifugation, resuspended in 10 ml of ice-cold phosphate-buffered saline (PBS: 50 mM NaH₂PO₄, Na₂HPO₄, 154 mM NaCl, pH 7.4) containing 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF) and aprotinin and leupeptin at 2 mg/ml each (Buffer A), and lysed by sonication. The particulate suspension was pelleted by centrifugation at 30,000g for 15 min at 4 °C. The clear supernatant was incubated with 2 ml of a 50% slurry of S-linked glutathione agarose (Pharmacia) for 20 min at 4 °C, and the agarose gel was collected in a column. After washing with 10 volumes of PBS supplemented with 10 mM EDTA, elution was achieved with 15 ml of 20 mM reduced glutathione in 50 mM Tris-HCl (final pH 8.0). The affinity-purified fusion protein was concentrated and dialysed against PBS before being used for subsequent immunizations. The integrity of the protein was verified by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE,³⁵) and 200 µg aliquots of the purified protein were frozen at -80 °C until their use for immunization.

Immunization of the rabbits and affinity purification of antisera

Four white New Zealand male rabbits were immunized with 0.2 mg affinity-purified fusion protein each month: the first two injections were done with the antigen emulsified in complete Freund's adjuvant (Difco). Subsequent booster injections were achieved with the antigen mixed with incomplete Freund's adjuvant. Blood samples were taken before each injection and clotted. The resulting sera were decomplemented by heating at 56 °C for 30 min, dialysed extensively against 0.9% NaCl (in order to remove endogenous 5-HT), and finally mixed with an equal volume of glycerol for storage at -30 °C.

The fusion protein (5-HT_{1A}i3 GST, 20 mg) or GST (20 mg) was conjugated to Affi-Gel 15 (Bio-Rad) according to the manufacturer's protocol. Affinity purification of antisera first consisted of passing the serum through the GST-Affi-Gel 15 column previously equilibrated with 25 mM Tris-HCl (pH 7.4). The effluent was then loaded on the 5-HT_{1A}i3 GST-Affi-Gel 15 column, which was subsequently washed with 20 ml of 25 mM Tris-HCl (pH 7.4) and eluted with 10 mM glycine-HCl (pH 2.0). Eluate fractions (2 ml each) were immediately neutralized, and those containing anti-5-HT_{1A}i3 GST specific antibodies (as assessed by ELISA, see below) were concentrated against PBS using a Micro-Pro-Dicon apparatus (Bio-Molecular Dynamics) at 4 °C. The final solution of antibodies was mixed with glycerol (up to 50%), and kept at -30 °C until use.

Purified anti-GST antibodies were obtained using the same protocol (washing and elution) applied to the GST-Affi-Gel 15 column after the serum sample had passed through.

Enzyme-linked immunosorbent assay

The fusion protein 5-HT_{1A}i3 GST or the synthetic peptide Gly²⁴-Glu²⁸ (see Fig. 1A, 10 µg/ml each), dissolved in 0.1 M sodium carbonate (pH 10.8), was added to each well of a microtiter plate, which was then incubated for 1 h at 37 °C. The wells were washed three times with PBS supplemented with 0.1% Tween-20 (PBST), filled with PBS containing fat-free milk (3%, wt vol), and incubated for 1 h at 37 °C before washing three times with PBST. Fifty microlitres of serially diluted antisera (10⁻¹ to 10⁻⁶ in PBST containing 3% fat-free milk) were then added to each well and the plate was incubated for 2 h at 37 °C. After washing three times with PBST, the secondary antibody, biotinylated goat anti-rabbit IgG (10⁻³ dilution in PBS supplemented with 3% fat-free milk), was added to each

well, and incubation proceeded for 1 h at 37°C, before extensive washing with PBST. Then, streptavidin peroxidase (10^{-3} diluted in PBST containing 3% fat-free milk) was added for 1 h at 37°C, and the wells were finally washed twice with PBST and once with PBS. The bound antibodies were detected by colour development following the addition of *O*-phenylenediamine dihydrochloride in 0.1 M sodium citrate containing 0.03% H_2O_2 (pH 5.5) to each well. The reaction was stopped by sulphuric acid (4 N final), 10 min later, and the absorbance was measured at 492 nm using a microplate reader (Sigma).

Immunoblotting

Fusion protein 5-HT_{1A}-i3-GST and free GST were electrophoresed in a 12% acryl/bisacrylamide slab gel in the presence of SDS.²² Proteins in the gel were transferred to a nitrocellulose sheet which was then preincubated for 1 h at room temperature in PBS containing 5% (wt/vol) fat-free dry milk, rinsed five times (10 min each) in PBS and finally incubated overnight at room temperature with anti-5-HT_{1A}-i3-GST or anti-GST antibodies (1:250 final dilution in PBS supplemented with 0.05% Tween-20 and 1% fat-free milk). After five washes with PBS, the nitrocellulose sheet was post-incubated for 1 h at room temperature in PBS containing 0.05% Tween-20, 1% fat-free milk and goat anti-rabbit IgG coupled to peroxidase (1:2000 dilution). The excess of the latter IgG was eliminated by extensive washing with PBS, and the nitrocellulose sheet was soaked in 50 mM sodium phosphate (pH 7.2) for 10 min. The bands with peroxidase activity were finally revealed by incubation in 50 mM sodium phosphate (pH 7.2) supplemented with 0.05% (wt/vol) 3,3'-diaminobenzidine tetrachloride (DAB), 0.02% $CoCl_2$, 0.02% NH_2Ni and 0.002% H_2O_2 . The coloured reaction was stopped by washing with water.

Immunoprecipitation of solubilized 5-hydroxytryptamine_{1A} receptors

Aliquots (0.3 ml) corresponding to ~1.6 mg protein) of the 100,000g supernatant of a suspension of rat hippocampal membranes preincubated for 60 min with 10 mM 3-[3-(choleamido-propyl)dimethylammonio]-1-propane sulphonate (CHAPS; see Ref. 10, for details) were mixed with 30 μ l of the antiserum diluted in 0.05 M Tris-HCl, pH 7.4 (final dilution: 1:50 to 1:1,000), and left overnight at 4°C. Other samples ("controls") were mixed with the preimmune serum instead of the antiserum, but at the same dilutions. The next day, the mixtures were supplemented with 70 μ l of a protein A-Sepharose CL-4B slurry (Pharmacia, diluted by half in 0.05 M Tris-HCl, pH 7.4), and then gently rotated for 2 h at 4°C. Finally, samples were centrifuged (2500 g, 10 min, 4°C), and the supernatants were saved for binding assays. Aliquots (70 μ l) of each supernatant were incubated with 1.0 nM [³H]-OH-DPAT in 0.05 M Tris-HCl, pH 7.4 (total volume: 0.2 ml) for 1 h at 15°C, and bound ³H was trapped on Whatman GF/B filters presoaked with 0.3% polyethylenimine as described previously.⁹ Non-specific [³H]-OH-DPAT binding was determined from similar samples supplemented with 10 μ M unlabelled 5-HT. Binding assays on aliquots (70 μ l) of supernatants exposed to the preimmune serum or anti-5-HT_{1A}-i3-GST antibodies were also performed with [³H]-5-HT, [³H]-mesulergine, [³H]-ketanserin, [³H]-zacopride, [³H]-prazosin and [³H]-dihydroalprenolol for the quantification of 5-HT_{1B}, 5-HT_{2C}, 5-HT_{2A}, 5-HT₃, α_1 and β -adrenergic receptors, respectively. The detailed protocols for these assays have previously been described.³⁹

Adenylate cyclase assays

Forskolin (10 μ M)-activated adenylate cyclase was assayed in rat hippocampal membranes by measuring the conversion of α -[³²P]ATP (0.1 mM) into [³²P]cyclic AMP in the presence of 0.1 M NaCl and 10 μ M GTP as described

in detail elsewhere.¹⁷ The inhibitory effect of 5-HT_{1A} receptor stimulation by a saturating concentration of 5-HT (1 μ M—see Ref. 8) was tested on native membranes and on those which had been preincubated for 1 h at 0°C with various dilutions (1:50, 1:100, 1:250) of the antiserum or the preimmune serum. Adenylate cyclase activity is expressed in nmol [³²P]cyclic AMP formed per mg membrane protein after a 20 min incubation at 30°C.¹⁷

Immunautoradiographic labelling of brain sections

Adult male Sprague-Dawley rats (250–300 g body weight) were anaesthetized with chloral hydrate (350 mg/kg i.p.) and perfused via the ascending aorta with 200 ml of 0.9% NaCl containing sodium nitrite (1 g/l). Animals were decapitated and the brain was quickly removed and frozen in isopentane at -30°C. Coronal and horizontal sections (thickness 20 μ m) were cut at -20°C and thaw-mounted on to gelatin-coated slides. After storage at -20°C for two weeks, sections were dipped in PBS supplemented with 4% paraformaldehyde for 3 min at room temperature (as for all the following steps), washed three times with PBS and then preincubated in PBS supplemented with 3% (wt/vol) bovine serum albumin (BSA) for 30 min. Then, the procedure was as described in detail elsewhere.¹⁷ Briefly, sections were washed 5 min in PBS and incubated with the crude antiserum (dilution 1:2000) or with the purified antiserum (dilution 1:1000) for 2 h. Controls were performed using preimmune serum, purified antibodies saturated by 5-HT_{1A}-i3-GST antigen (100 μ g/ml) or anti-GST antibodies (1:2000 final dilution). All antiserum solutions were made in PBS supplemented with 1% BSA. After washing (three times for 10 min in PBS), sections were dipped in PBS supplemented with [³⁵S]IgG-anti-rabbit IgG (1.0 μ Ci/ml) for 2 h. They were then washed three more times in PBS for 10 min each, once in distilled water for 15 s, dried with cold air and exposed to β max films (Amersham) for three days. Autoradiograms were finally developed in Kodak Microdol (10 min at 20°C).

Immunohistochemical labelling of brain sections

Adult male Sprague-Dawley rats (250–300 g body weight) were anaesthetized with chloral hydrate (350 mg/kg i.p.) and perfused intracardially with 100 ml of 0.9% NaCl followed by 300 ml of 100 mM sodium phosphate buffer (PB, pH 7.4) supplemented with 4% paraformaldehyde. The brains were removed, postfixed by immersion in the same fixative for 60 min, and cryoprotected in PB containing 30% sucrose and 0.1% sodium azide for 48 h at 4°C. Brains were then sectioned at 35 μ m with a sliding cryomicrotome, and sections were collected in PBS. Sections were pretreated for 10 min at 4°C with 2% H_2O_2 in PBS (pH 7.4) washed twice in the same buffer for 10 min, then preincubated in PBS containing 3% BSA and 0.25% Triton X-100 for 30 min at 4°C, washed in PBS and finally incubated overnight at 4°C with the purified anti-5-HT_{1A}-i3-GST antibodies (final dilution 1:1000) in PBS containing 1% BSA and 0.25% Triton X-100. Control experiments consisted of replacing the antibody solution by the preimmune serum at the same dilution or the antibodies previously saturated by the fusion protein (after an incubation for 1 h at room temperature with 0.1 mg 5-HT_{1A}-i3-GST per ml of the solution of purified antibodies). After washing twice in PBS for 10 min, sections were incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector) diluted at 1:250 in PBS containing 1% BSA and 0.25% Triton X-100, washed and exposed to avidin-biotin-horseradish peroxidase complex (ABC, Vector, dilution 1:200) for a further 1 h. The immunoperoxidase reaction then proceeded by incubating the sections in 0.02% DAB and 0.002% H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 10–15 min. Sections were finally rinsed, mounted on gelatin-coated slides, dehydrated and coverslipped for light microscopy and photomicrography.

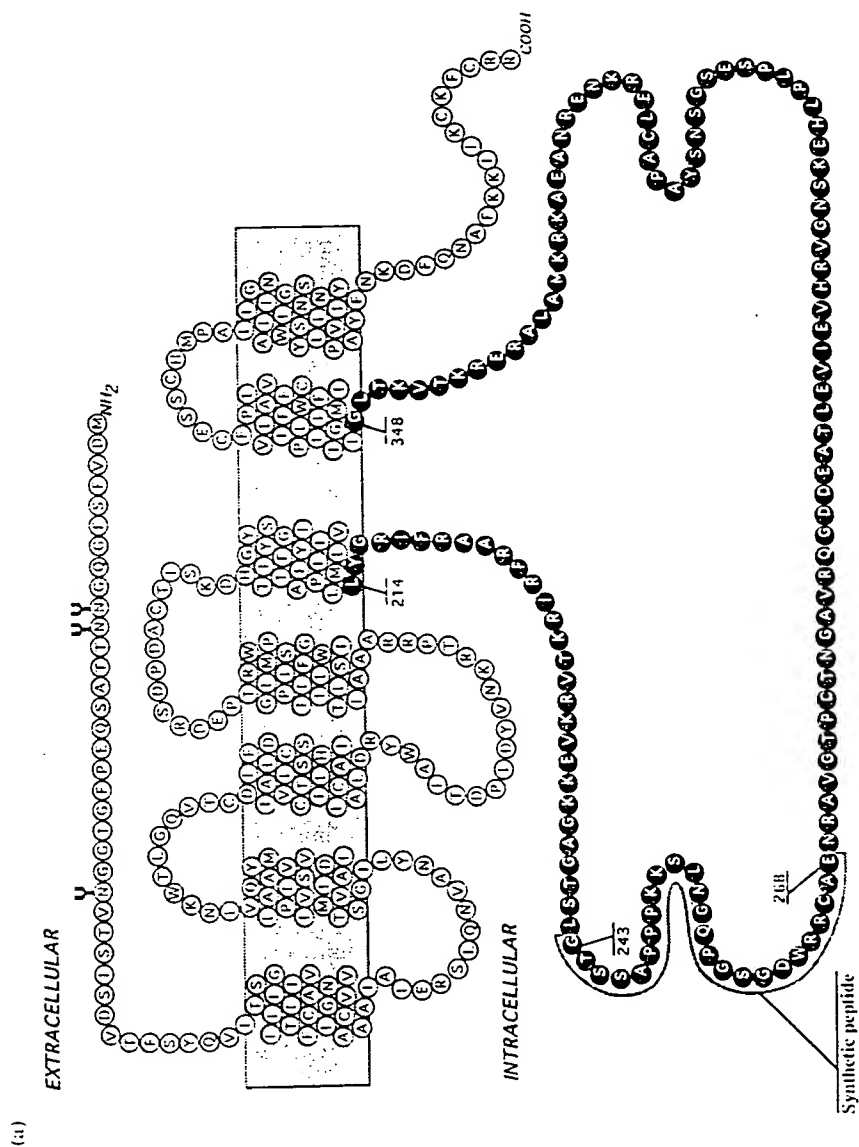


Fig. 1. (a) Schematic representation of the amino acid sequence of the rat 5-HT_{1A} receptor (from Ref. 2). The portion between Leu³⁸⁴ and Gly³⁹⁶ (sequence of 135 aa in black) was selected to be coupled with GST for the construction of the fusion protein 5-HT_{1A}-13-GST. The sequence Gly³⁹⁷-Glu⁴⁰⁶ in 13 is that of the 26 aa synthetic peptide that was used as antigen for raising anti-5-HT_{1A} receptor antibodies in a previous study.⁹ (b) Comparison of the aa sequence of 13 of 11 different 5-HT receptors coupled to G proteins. Amino acid residues of the 5-HT_{1A} receptor also found in other receptors are in grey boxes. These sequences are from Ref. 2, rat 5-HT_{1A}; Ref. 48, rat 5-HT_{1B}; Ref. 16, rat 5-HT_{1D}; Ref. 50, human 5-HT_{1D}; Ref. 33, human 5-HT_{1C}; Ref. 1, human 5-HT_{1F}; Ref. 21, rat 5-HT_{1F}; Ref. 41, rat 5-HT_{1T}. The nomenclature adopted for these receptors is that recommended by the IUPHAR Committee for Receptor Classification and Drug Nomenclature.²⁰

Chemicals

[³H]-8-hydroxy-2-(α -*n*-propylamino) tetralin ([³H]-8-OH-DPAT, 100 Ci mmol) was from the Service des Molécules Marquées, CEA (91191 - Gif-sur-Yvette, France). [³⁵S] goat IgG-anti-rabbit IgG (570 Ci mmol) and α -[³²P]ATP (20–30 Ci mmol) were from Amersham (U.K.). Other radioactive molecules were: [³H]-5-HT (12.5 Ci/mmol, Amersham, U.K.), [³H]-mesulergine (75.8 Ci mmol, Amersham, U.K.), [³H]-prazosin (25.4 Ci mmol, Amersham, U.K.), [³H]-dihydroalprenolol (49.4 Ci mmol, Amersham, U.K.), [³H]-ketanserine (New England Nuclear, Wilmington, U.S.A.) and [³H]-zacopride (82 Ci mmol, generously given by Delalande-Synthelabo Laboratories, Rueil-Malmaison, France). The 26 aa peptide Gly²⁴³–Glu²⁶⁸ was synthesized by Neosystem (Strasbourg, France). (–)-WAY 100135 [*N*-1-butyl-3,4-(2-methoxy-phenyl)piperazine-1-yl-2-phenyl propanamide dihydrochloride] was generously donated by Wyeth Labs (Taplow, U.K.). Sources of other compounds were: deoxynucleotide primers for PCR, Appligene (Illkirch, France); XhoI, Bam HI and T₄ DNA ligase, Biolabs (Montigny-le Bretonneux, France); phenylmethylsulphonyl fluoride (PMSF) and 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), Tebu (Le Perray-en-Yvelines, France); forskolin, Calbiochem (Los Angeles, CA, U.S.A.); leupeptin, aprotinin, isopropylthiogalactoside (IPTG), *O*-phenylenediamine dihydrochloride, polyethylenimine, biotinylated goat anti-rabbit IgG, streptavidin peroxidase, goat anti-rabbit IgG coupled to peroxidase, Sigma (St Louis, Mo, U.S.A.).

All other compounds were the purest commercially available (Prolabo, Merck, Boehringer Mannheim, Pharmacia).

RESULTS

Production of the 5-hydroxytryptamine_{1A}-i3 glutathione S-transferase fusion protein

As illustrated in Fig. 1a, the sequence that we selected for making the 5-HT_{1A}-i3 GST fusion protein corresponded to the entire third intracytoplasmic loop of the rat 5-HT_{1A} receptor from Leu²¹⁴ to Gly²⁴³. This rather hydrophilic 135 aa sequence is very specific of the rat 5-HT_{1A} receptor, as there are only 4–16% of conserved aa residues within the third intracytoplasmic loop of this receptor as compared with that of 10 other G-protein-coupled 5-HT receptors whose sequences are available to date (Fig. 1b). Most of these conserved aa residues are located at the C- and N-terminal parts of the i3 region. Thus, this region was a good candidate for the immunization of rabbits to raise specific anti-5-HT_{1A} receptor antibodies.

The 5-HT_{1A}-i3 GST fusion protein was expressed in *E. coli* and purified on a glutathione-agarose column. When extracts from bacteria not exposed to IPTG were applied on the column, there was no visible band on a SDS PAGE from the eluate (Fig. 2A, lane 2). In contrast, after IPTG induction, a major 43,000 band was seen from the glutathione affinity column eluate (Fig. 2A, lane 3). This band migrated to a position closely corresponding to the calculated molecular weight of the fusion protein: 42,400 (14,900 for the 5-HT_{1A}-i3 plus 27,500 for the GST). Smaller minor bands were also present on the SDS PAGE gel, but none of them migrated like free GST (Fig. 2A, lane 4). They

probably correspond to degradation products of the 5-HT_{1A}-i3 GST fusion protein.

Detection of anti-5-hydroxytryptamine_{1A}-i3/glutathione S-transferase antibodies by enzyme-linked immunosorbent assay

The material produced by IPTG-exposed bacteria and purified on the glutathione-agarose column was then used to immunize rabbits, and collected sera were screened for antibody detection by enzyme-linked immunosorbent assay (ELISA). As expected (Fig. 3A), preimmune serum very poorly recognized the 5-HT_{1A}-i3 GST fusion protein. In contrast, a very intense, dilution-dependent, colourimetric reaction was obtained with the serum collected one month after the third injection of the antigen (Fig. 3A). The serum dilution yielding half of the maximal colourimetric intensity could be calculated from curves such as that illustrated in Fig. 3A, and the –log of this value was considered as the titer of a given antiserum. Figure 3B shows the time course of a typical immune response. Anti-fusion protein antibodies were detected in the serum as early as one month after the first injection. Antibody production reached its maximum at the third injection and remained stable until the end of the immunization protocol.

In a previous report,⁹ we described an antiserum raised against a 26 aa synthetic peptide corresponding to the portion from Gly²⁴³ to Glu²⁶⁸ in the third intracellular loop of the rat 5-HT_{1A} receptor (see Fig. 1A). Taking advantage of the ELISA technique, we looked for possible cross-reactivity between anti-5-HT_{1A}-i3 GST fusion protein antibodies and these anti-peptide antibodies. The data in Fig. 4A show that both the anti-peptide and the anti-fusion protein antisera bound to the 5-HT_{1A}-i3/GST antigen with approximately the same titer. However, ELISA tests with the 26 aa synthetic peptide as antigen indicated that only the anti-peptide antibodies bound to it with a high titer. In contrast, the anti-fusion protein antibodies recognized this antigen very poorly (Fig. 4B).

Detection of anti-5-hydroxytryptamine_{1A}-i3/glutathione S-transferase antibodies by western blotting

For these experiments, 1 μ g of 5-HT_{1A}-i3 GST fusion protein or free GST were boiled in the presence of SDS, subjected to SDS-PAGE and the electrophoresed materials transferred onto a nitrocellulose sheet. The sera collected from all rabbits after the second injection of the fusion protein detected both free GST (lane 1, Fig. 2B) and a major band at 43,000 (lane 2, Fig. 2B). The typical western blot shown in Fig. 2B reveals that in addition to these materials, minor bands were also recognized by the anti-5-HT_{1A}-i3/GST antibodies. The molecular weights of the latter bands in fact corresponded to those of the minor bands, already detected by SDS-PAGE, of the material produced by

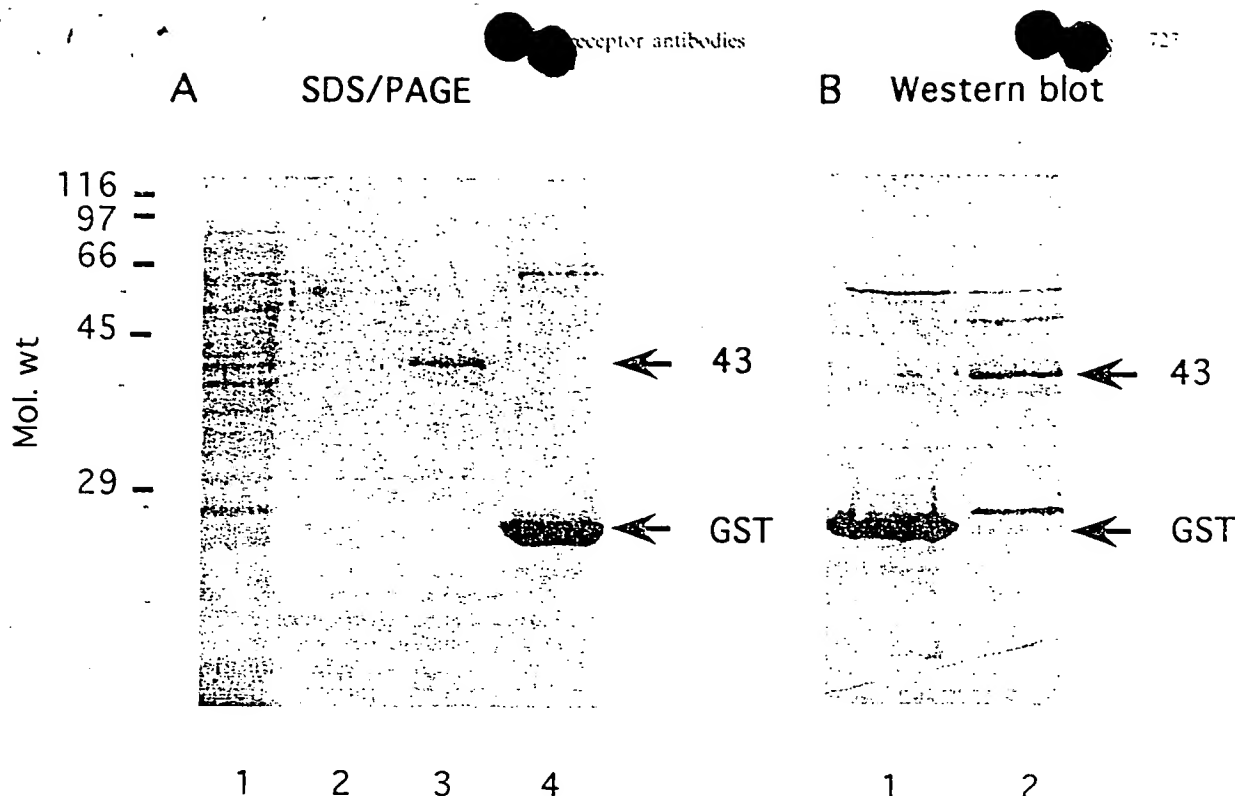


Fig. 2. SDS-PAGE (A) and western blot (B) of GST and of the material synthesized by *E. coli* transformed with the plasmid pGEX-KG and exposed, or not, to IPTG. Molecular weight is given $\times 10^{-3}$. (A) SDS-PAGE: lane 1, crude supernatant (30,000 g, 15 min, 4°C, $\sim 10 \mu\text{g}$ of protein) of an homogenate of transformed *E. coli* exposed to IPTG; lane 2, material eluted from the glutathione-agarose affinity column charged with the 30,000 g supernatant from transformed *E. coli* not exposed to IPTG; lane 3, eluted material ($1 \mu\text{g}$ protein) from transformed *E. coli* exposed to 0.1 mM IPTG; lane 4, $\sim 5 \mu\text{g}$ GST. Colouration was with Coomassie Blue. (B) Western blot of GST (lane 1) or of the material from transformed *E. coli* exposed to 0.1 mM IPTG (lane 2). Immunolabelling was achieved with purified anti-GST antibodies (lane 1) or purified anti-5-HT_{1A}-i3 GST antibodies (lane 2) from a rabbit serum collected one month after the third booster injection of the fusion protein antigen. Similar results were obtained with antisera collected from the four rabbits immunized with the fusion protein antigen. The band at 43,000 corresponds to the complete 5-HT_{1A}-i3 GST fusion protein.

IPTG-exposed bacteria and purified on the glutathione-agarose affinity column (lane 3, Fig. 2A). Attempts to detect the native 5-HT_{1A} receptors solubilized from rat hippocampal membranes by western blotting were unsuccessful. Indeed, the paucity of this receptor in the solubilisates made it undetectable by western blotting.

Immunoprecipitation of solubilized 5-hydroxytryptamine_{1A} receptors

The anti-5-HT_{1A}-i3/GST antibodies exerted only a minor effect on the specific binding of [³H]8-OH-DPAT to 5-HT_{1A} receptors solubilized by CHAPS from rat hippocampal membranes. Thus, only a 15% decrease in this binding was noted with solubilisates which had been incubated with the antiserum at 1:50–1:100 final dilution for 16 h at 4°C (Fig. 5). However, addition of protein A-Sepharose CL-4B beads followed by centrifugation resulted in a dramatic dilution-dependent reduction of 5-HT_{1A} binding sites from the supernatant (Fig. 5). In contrast, no decrease in the specific binding of [³H]8-OH-DPAT was noted in solubil-

isates exposed to preimmune serum in the absence or presence of protein-Sepharose CL-4B. In addition, the specific binding of [³H]5-HT to 5-HT_{1B} sites, [³H]mesulergine to 5-HT_{2C} sites, [³H]ketanserin to 5-HT_{2A} sites, [³H]zacopride to 5-HT₃ sites, [³H]prazosin to α_1 -adrenergic sites and [³H]dihydroalprenolol to β -adrenergic sites in solubilisates from rat hippocampal membranes was not significantly different whether the latter preparations were exposed or not to anti-5-HT_{1A}-i3/GST antibodies with or without protein A-sepharose CL-4B beads (not shown).

Maximal immunoprecipitation (I_{max} , $\sim 80\%$) of 5-HT_{1A} binding sites solubilized from rat hippocampal membranes was obtained with a 1:50 dilution of antiserum, and half maximal immunoprecipitation was achieved with the antiserum at $\sim 1:350$. If one considered the $-\log$ of this value as a second titer estimate of the antiserum, it was also possible to follow the appearance of anti-5-HT_{1A}-i3/GST antibodies during the immunization protocol. As illustrated in Fig. 6A, antibodies able to immunoprecipitate solubilized 5-HT_{1A} binding sites were

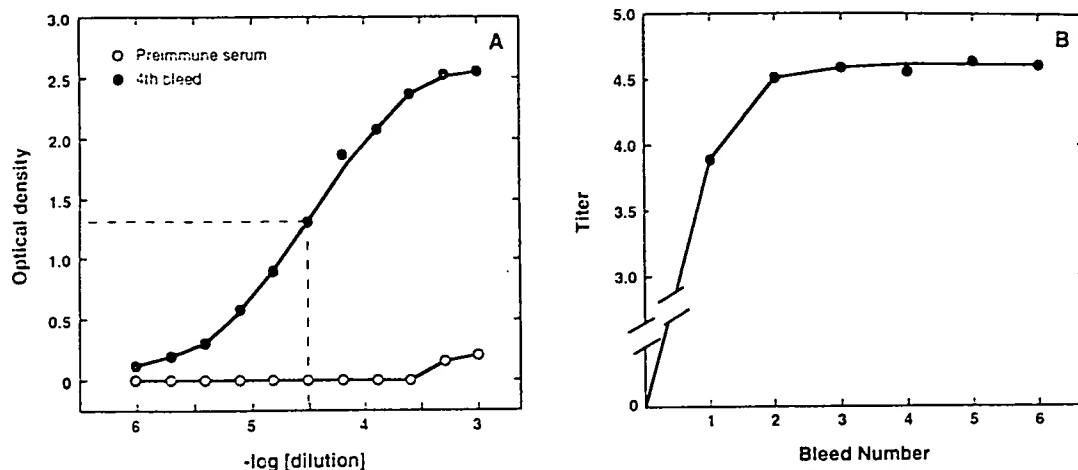


Fig. 3. ELISA detection and titration of anti-5-HT_{1A}-i3 GST antibodies. (A) ELISA: Dilution-dependent colour reaction (optical density was measured at 492 nm) showing the presence of antibodies to the 5-HT_{1A}-i3 GST fusion protein in the serum from the fourth bleed of an immunized rabbit. Similar curves were obtained with the four rabbits included in the study. In contrast, no colour developed with preimmune serum, as expected from the lack of anti-5-HT_{1A}-i3 GST antibodies. (B) Time-course evolution of the titer of anti-5-HT_{1A}-i3 GST antibodies: The titer is expressed as $-\log$ of the serum dilution required to yield half maximal OD at 492 nm in ELISA (see A). Similar curves were obtained with the four rabbits immunized with the fusion protein antigen.

already present in the rabbit serum one month after the first injection of the 5-HT_{1A}-i3 GST fusion protein. However, at this stage, only ~20% of solubilized 5-HT_{1A} sites could be immunoprecipitated, at maximum, by the antiserum (at 1:20–1:50, final

dilutions) (Fig. 6B). Thereafter, both the immunoprecipitating capacity and the titre of the antiserum increased to their maximal values, which were reached at the third bleeding and then plateaued up to the end of the immunization protocol (Fig. 6A, B).

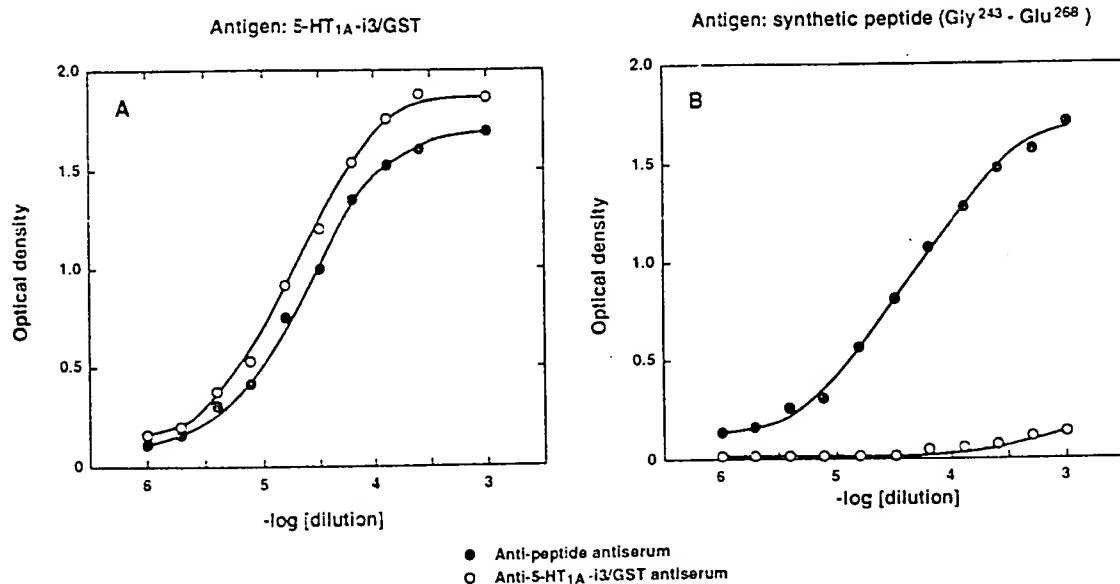


Fig. 4. Cross reactivity of anti-5-HT_{1A}-i3 GST antibodies and anti-Gly²⁴³-Glu²⁶⁸ antibodies.⁹ (A) ELISA with the 5-HT_{1A}-i3 GST protein as antigen. Both the serum from the fourth bleed or a rabbit immunized with this material (○), and the antiserum⁹ raised against the Gly²⁴³-Glu²⁶⁸ sequence of the rat 5-HT_{1A} receptor² (●) yielded a dilution-dependent colour reaction (OD was measured at 492 nm), indicating that they bound to the antigen (with a similar titer). Similar observations were made with antisera from the four rabbits immunized with the 5-HT_{1A}-i3 GST fusion protein. (B) ELISA with the synthetic peptide corresponding to the Gly²⁴³-Glu²⁶⁸ portion of the rat 5-HT_{1A} receptor as antigen. In this case, only the anti-peptide antiserum (●) yielded a colour reaction. In contrast, the serum from rabbits immunized with the 5-HT_{1A}-i3 GST fusion protein (○) did not recognize the synthetic peptide. These typical curves were obtained with sera from the four rabbits included in the study.

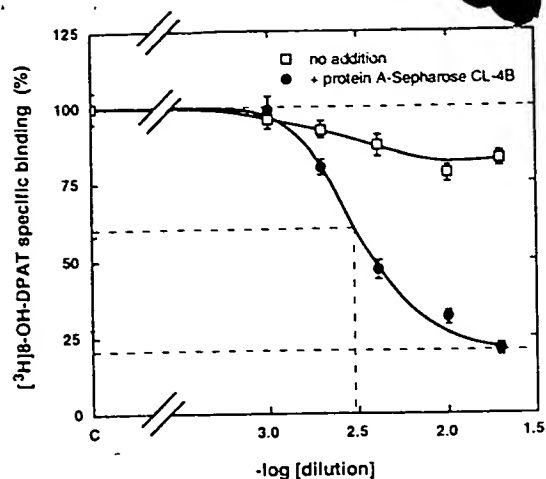


Fig. 5. Immunoprecipitation of 5-HT_{1A} receptor binding sites solubilized from rat hippocampal membranes. Aliquots (0.3 ml) of a solubilise from rat hippocampal membranes¹⁰ were incubated overnight at 4°C with 30 μ l of 0.05 M Tris-HCl, pH 7.4 (C on abscissa) or 30 μ l of 1:50–1:1000 dilutions (in the same buffer; abscissa) of the serum from the fourth bleed of a rabbit immunized with the 5-HT_{1A}-i3/GST fusion protein. Binding assays were carried out with 1 nM [³H]8-OH-DPAT either directly on aliquots (70 μ l) of each mixture (□) or on aliquots (70 μ l) of supernatants after the addition of protein A-Sepharose CL-4B to each mixture (●). [³H]8-OH-DPAT specific binding is expressed as a percentage of that found with solubilisates not supplemented with serum (C on abscissa: 100% = 219 or 234 fmol/ml of solubilise exposed or not to protein A-Sepharose CL-4B, respectively). Each point is the mean \pm S.E.M. of triplicate determinations in three independent experiments. The $-\log$ of serum dilution producing half maximum reduction of [³H]8-OH-DPAT specific binding in samples supplemented with protein A-Sepharose CL-4B is considered as the titre of antibodies for immunoprecipitation of solubilized 5-HT_{1A} receptor binding sites.

Effects of anti-5-hydroxytryptamine_{1A}-i3 glutathione S-transferase antibodies on the coupling of 5-hydroxytryptamine_{1A} sites to G protein and adenylate cyclase

In agreement with previous observations,¹¹ GppNHp was found to decrease, in a concentration-dependent manner, the specific binding of [³H]8-OH-DPAT to 5-HT_{1A} sites solubilized from rat hippocampal membranes down to ~20% of its control value, with an IC_{50} of $0.60 \pm 0.13 \mu$ M (mean \pm S.E.M., $n = 3$). Preincubation of membrane solubilisates with preimmune serum or the antiserum (fourth bleeding) at a final dilution of 1:50 for 1 h at 0°C did not significantly affect the potency of GppNHp to decrease the specific binding of [³H]8-OH-DPAT (IC_{50} of $0.54 \pm 0.13 \mu$ M with the preimmune serum; $0.64 \pm 0.14 \mu$ M with the antiserum; means \pm S.E.M., $n = 3$). Similar observations were made with hippocampal membranes exposed to the preimmune serum or the antiserum under the same conditions as those used for solubilisates, and then tested for [³H]8-OH-DPAT binding (not shown).

As expected from the negative coupling of 5-HT_{1A} receptors with adenylate cyclase, 5-HT (1 μ M)

reduced by ~20% the forskolin-stimulated enzyme activity in rat hippocampal membranes (Table 1), and this effect could be completely prevented by the selective 5-HT_{1A} receptor antagonist (+)N-t-butyl-3,4-(2-methoxyphenyl)piperazin-1-yl-2-phenylpropanamide [(+)-WAY 100135²²] at 1 μ M (not shown). Interestingly, no change in forskolin-stimulated adenylate cyclase activity in the absence as well as in the presence of 1 μ M 5-HT was noted after the preincubation of rat hippocampal membranes with various dilutions (1:50–1:250) of the preimmune serum or the antiserum for 1 h at 0°C (Table 1).

Immunautoradiographic labelling by anti-5-hydroxytryptamine_{1A}-i3 glutathione S-transferase antibodies

Labelling by [³⁵S]IgG-anti-rabbit IgG of anti-5-HT_{1A}-i3/GST antibodies bound to brain sections revealed the same distribution as that of 5-HT_{1A} receptor binding sites autoradiographically labelled by specific radioligands.³⁷ In particular, the septal area, dentate gyrus and CA1 area of the hippocampus, entorhinal cortex and dorsal raphe nucleus were all recognized by the anti-5-HT_{1A}-i3 GST antiserum (Fig. 7). In contrast, the striatum, substantia nigra, cerebellum and choroid plexus did not bind the antiserum. Nowhere in brain sections made at the level of the septum, hippocampus and dorsal raphe nucleus, could immunautoradiographic labelling above background noise be detected when anti-5-HT_{1A}-i3 GST antibodies were replaced by either the preimmune serum (Fig. 8), anti-5-HT_{1A}-i3/GST antibodies saturated by the fusion protein or purified anti-GST antibodies (not shown). In contrast, similar immunautoradiograms were obtained whether anti-5-HT_{1A}-i3 GST antibodies were previously saturated or not by GST (by a pretreatment with 0.1 mg pure GST/ml of antiserum for 1 h at room temperature; Fig. 8).

Immunohistochemical staining by anti-5-hydroxytryptamine_{1A}-i3 glutathione S-transferase antibodies

Immunoperoxidase activity due to the binding of anti-5-HT_{1A}-i3/GST antibodies superimposed exactly onto the immunautoradiographic labelling noted above at all levels of the rat brain examined (not shown). Particular attention was paid to the septal area, the hippocampus and the interpeduncular nucleus, where 5-HT_{1A} receptors are located postsynaptically on neuronal targets of serotonergic neurons,⁴⁷ and the anterior raphe nuclei, where these receptors are on the somas and dendrites of the latter neurons, and act as autoreceptors.^{37,47}

At the anterior level of the septum, brain sections exhibited a strong immunostaining within the dorsal nucleus and the intermediate nucleus of the lateral septum, whereas the caudate-putamen was essentially devoid of immunohistochemical labelling (Fig. 9A). Higher magnification showed that the immunostaining was exclusively associated with the neuropile in the lateral septum (Fig. 10B), but cell

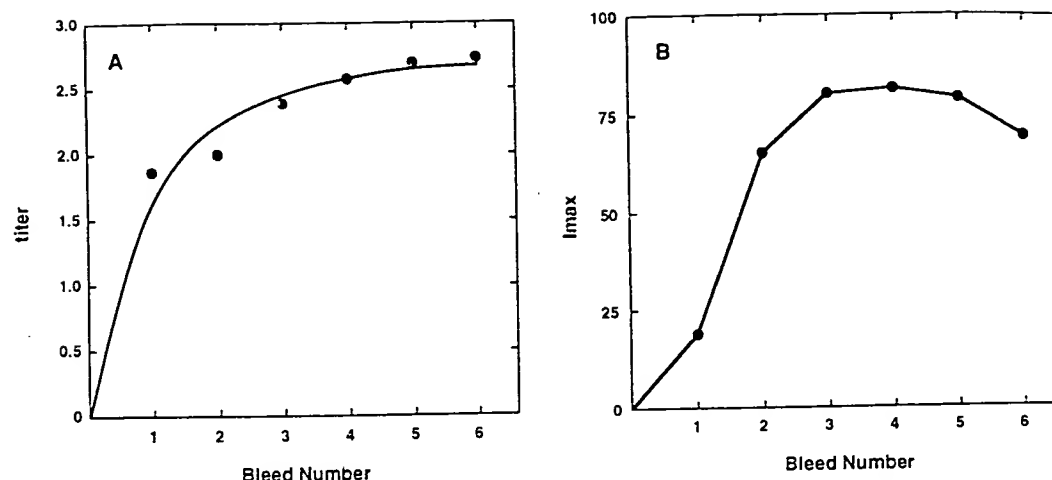


Fig. 6. Time-course evolution of the capacity of antisera to immunoprecipitate 5-HT_{1A} receptor binding sites solubilized from rat hippocampal membranes. (A) Evolution of the titer of antibodies for immunoprecipitation of solubilized 5-HT_{1A} receptor binding sites during the immunization protocol. Experiments were as described in the legend to Fig. 5 for samples supplemented with protein A-Sepharose CL-4B. This curve is typical of those obtained with the four rabbits immunized with the 5-HT_{1A}-i3 GST fusion protein antigen. (B) Evolution of the maximal capacity of antibodies to immunoprecipitate solubilized 5-HT_{1A} receptor binding sites during the immunization protocol. *I*_{max} (ordinate) corresponded to the maximal reduction of [³H]8-OH-DPAT specific binding in solubilisates exposed to 1:20–1:50 dilutions of serum and protein A-Sepharose CL-4B (see Fig. 5). Similar data were obtained with the four rabbits injected each month with the 5-HT_{1A}-i3 GST fusion protein.

bodies whose plasma membranes were endowed with positive immunostaining were observed in the median septum and the nucleus of the vertical limb of the diagonal band (Fig. 11B).

Within the hippocampal area, the immunostaining was uneven (Fig. 9B) with the dentate gyrus showing

an intense labelling, particularly in the inner blade. The CA1 area of Ammon's horn was also strongly reactive, with the stratum oriens exhibiting a slightly higher level of labelling than the stratum radiatum and the stratum lacunosum moleculare (Fig. 10A). In contrast, the CA2 area was nearly devoid of immunolabelling, and the CA3 area was faintly stained (Fig. 10A). In all zones of the hippocampus, the immunolabelling was restricted to the dendritic areas, where it appeared as a dense and homogeneous staining. Cell somas were not stained as shown by the lack of immunohistochemical reaction at the level of both the pyramidal cell layer in the CA1 area and the granular cell layer in the dentate gyrus (Fig. 10A). However, a few labelled cells bodies were occasionally found in the CA2 area (not shown).

Within the interpeduncular nucleus, the immunohistochemical labelling by anti-5-HT_{1A}-i3 GST antibodies was also heterogeneous with a very intense staining of the dorsolateral subnucleus (Fig. 10C). The rostral subnucleus also exhibited a rather high immunohistochemical reaction, whereas the ventral area within the interpeduncular nucleus (i.e. the caudal subnucleus and lateral subnucleus) was immunostained only to a moderate level (Fig. 10C). As observed in the lateral septum and the CA1 area of the hippocampus, immunostaining of the various subregions of the interpeduncular nucleus exclusively concerned the neuropile with no immunostained cell bodies.

At the level of the anterior raphe nuclei, the dorsal raphe nucleus exhibited dense immunostaining, and the median raphe nucleus was also labelled, though

Table 1. Basal and 5-hydroxytryptamine-modulated adenylate cyclase activity in rat hippocampal membranes exposed to preimmune serum, anti-5-hydroxytryptamine-i3 glutathione *S*-transferase antiserum or none

Pretreatment	[³ P]cyclic AMP (nmol/mg protein)		
	Basal	5-HT (1 μ M)	%
None	0.95 \pm 0.02	0.78 \pm 0.01	-18
Preimmune serum			
1:50	0.98 \pm 0.02	0.77 \pm 0.01	-21
1:100	0.96 \pm 0.01	0.77 \pm 0.02	-20
1:250	0.95 \pm 0.02	0.76 \pm 0.01	-20
Antiserum			
1:50	1.01 \pm 0.02	0.81 \pm 0.02	-20
1:100	0.95 \pm 0.03	0.79 \pm 0.02	-17
1:250	0.97 \pm 0.02	0.76 \pm 0.01	-22

Freshly prepared rat hippocampal membranes were incubated for 1 h at 0°C with preimmune serum or anti-5-HT_{1A}-i3 GST antiserum (fourth bleed) at 1:50–1:250 final dilution or "none" (i.e. with only glycerol, at the same concentration, 1% v/v, as that added with the lowest dilution, 1:50, of each serum). Adenylate cyclase assays were then performed in the presence of 10 μ M forskolin, 10 μ M GTP and 0.1 M NaCl as described in Experimental Procedures. Each value is the mean \pm S.E.M. of [³P]cyclic AMP (in nmol/mg protein) formed after a 20 min incubation at 30°C in the presence or the absence ("basal") of 1 μ M 5-HT. Whatever was the membrane pretreatment, no significant difference was noted on the percent decrease (%) in [³P]cyclic AMP formation due to 1 μ M 5-HT.

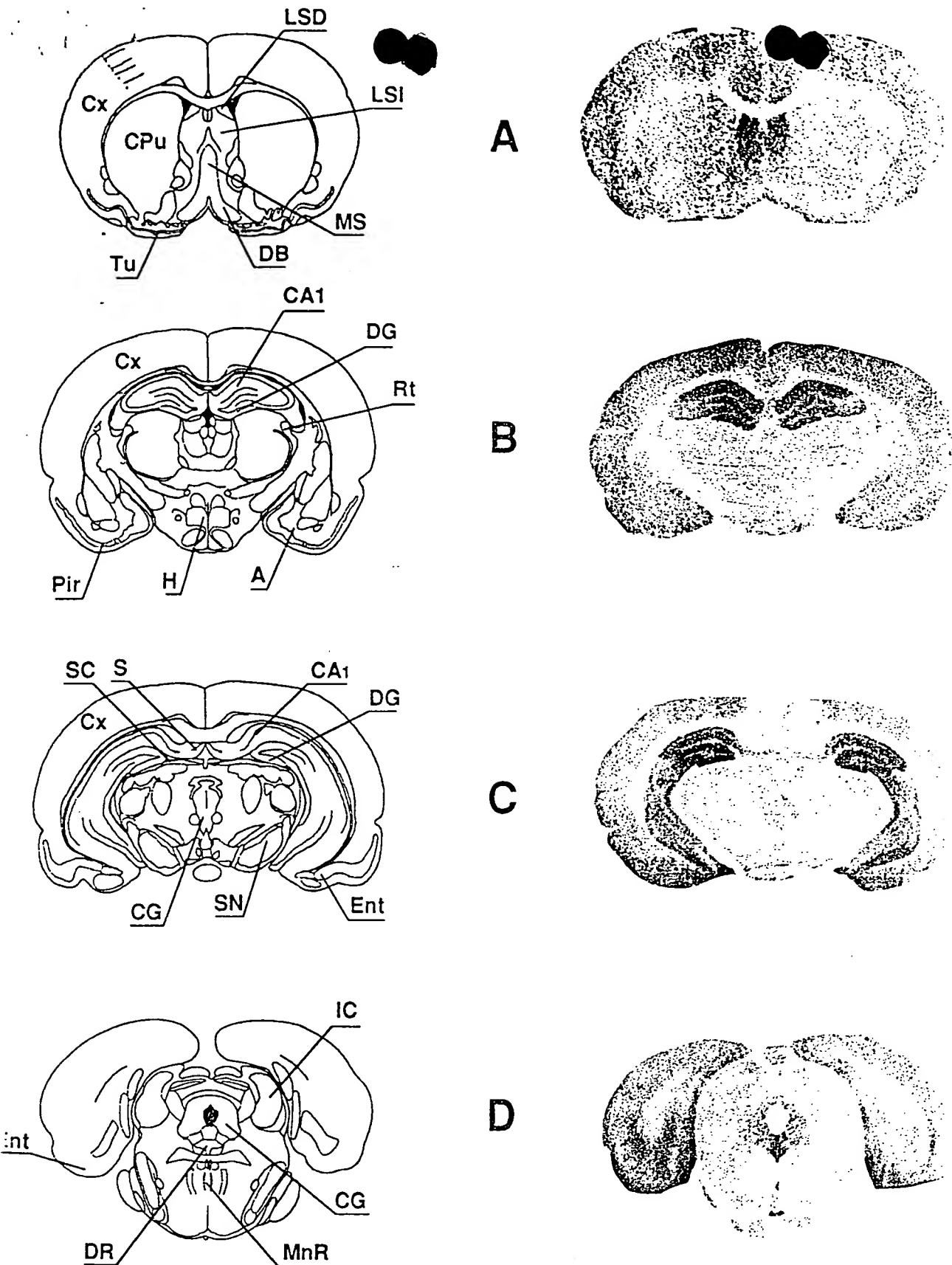


Fig. 7. Immunohistochemical staining of rat brain coronal sections exposed to purified anti-5-HT_{1A}-i3 GST antibodies. Sections (20 μ m thick) along the anterior-posterior axis (from A to D) show that the septum (A), hippocampus (B,C), entorhinal cortex and dorsal raphe nucleus (D) bound these antibodies (at 1:1000 dilution). LSD, dorsal part of the lateral septum; LSI, intermediate part of the lateral septum; MS, medial septum; Cx, cerebral cortex; CPu, caudate-putamen; DB, diagonal band of Broca; Tu, olfactory tubercle; CA1, CA1 area of Ammon's horn; DG, dentate gyrus; Rt, reticular nucleus of the thalamus; A, amygdala; H, hypothalamic nuclei; Pir, piriform cortex; S, dorsal subiculum; SC, superior colliculi; CG, central gray area; SN, substantia nigra; Ent, entorhinal cortex; IC, inferior colliculi; DR, dorsal raphe nucleus; MnR, median raphe nucleus.

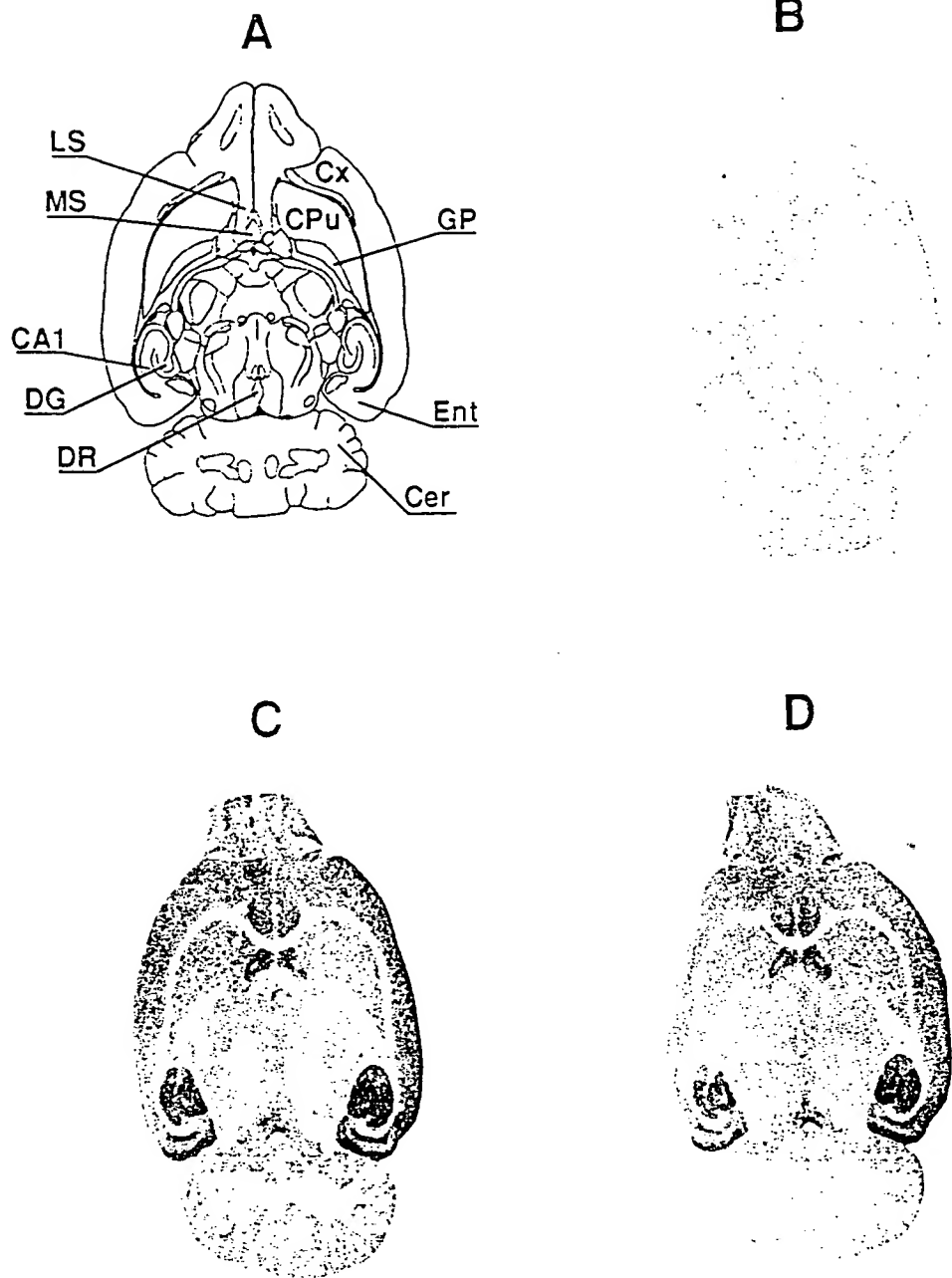


Fig. 8. Immunohistochemical staining of horizontal rat brain sections exposed to preimmune serum (B), purified anti-5-HT_{1A}-i3 GST antibodies (B) or purified anti-5-HT_{1A}-i3 GST antibodies saturated with GST (C). Antibodies were used at 1:1000 dilution. LS, lateral septum; MS, medial septum; GP, globus pallidus; Cer, cerebellum. Other abbreviations are as indicated in the legend to Fig. 7.

less strongly (Fig. 9C). In addition, some immunoreactivity was observed in the superior colliculus (superficial gray layer) and in the central gray (Fig. 9C). Numerous cell bodies exhibited positive immunostaining at their periphery within both median (Fig. 11A) and dorsal (not shown) raphe nuclei. In most cases, the labelling was continuous and outlined the whole perikaryon, but patches of more intense immunolabelling were also observed (Fig. 11A). The same subcellular distribution of

immunoreactivity was observed in the zone incerta (Fig. 11C) where both cell types with continuous or patchy immunostaining all around the perikaryon could be found (Fig. 11C).

In addition to the perikaryal surface reactivity, immunoprecipitate was also associated with dendrites in both the median and raphe nuclei. As shown in Fig. 11A, some dendrites could be visualized on a rather long distance, thanks to a thin uneven staining that gave them a straw-like appearance.

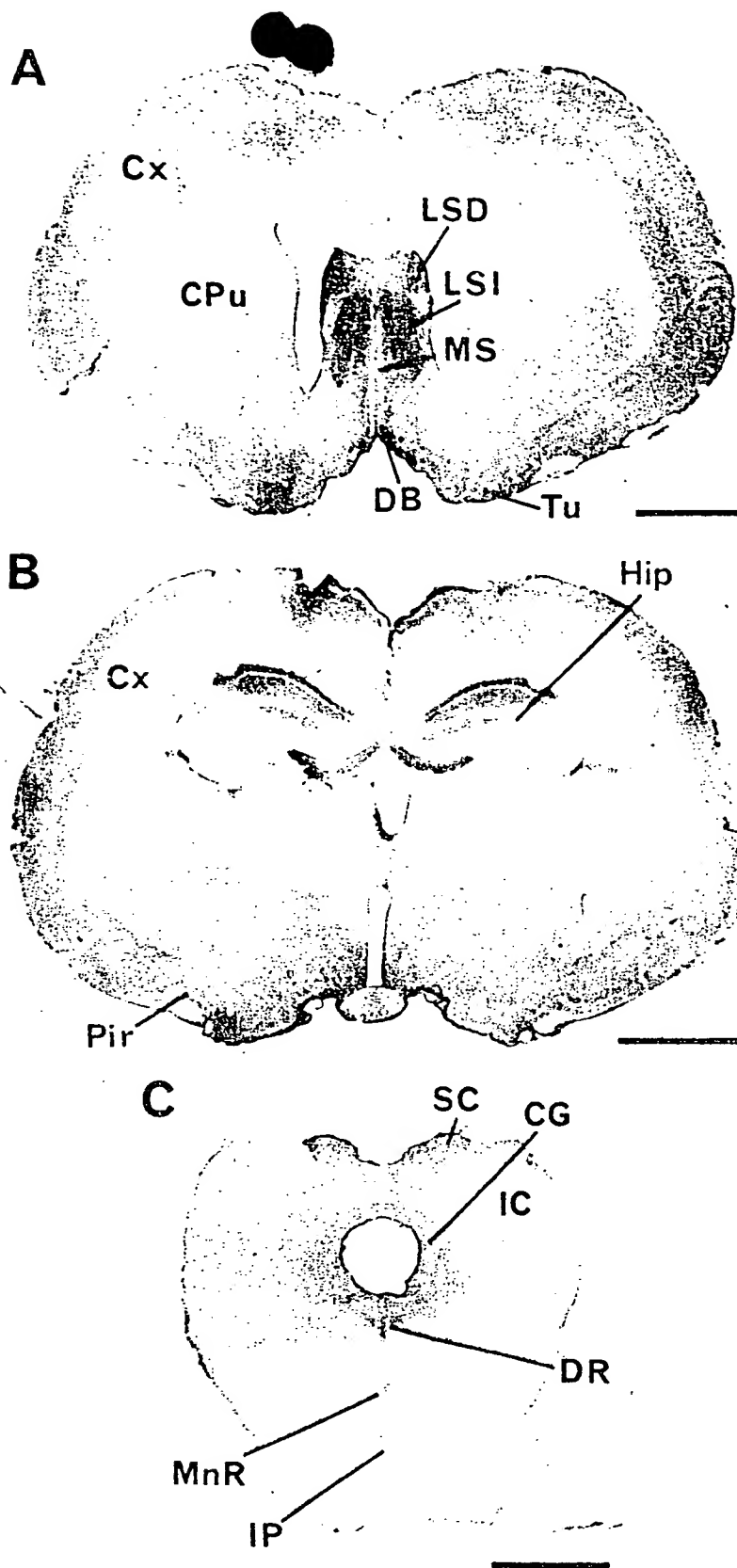


Fig. 9. Immunohistochemical staining with purified anti-5-HT_{1A}-i3/GST antibodies showing the three brain areas with the most intense immunoreactivity. Purified anti-5-HT_{1A}-i3/GST antibodies were used at 1:1000 final dilution. (A) Frontal section at the level of the septum with intense immunolabelling in the dorsal nucleus (LSD) and the intermediate nucleus (LSI) of the lateral septum. Cx, cerebral cortex; CPu, caudate putamen; MS, medial septum; DB, diagonal band; Tu, olfactory tubercle (scale bar, 2 mm). (B) Frontal section at the level of the dorsal hippocampus (Hip). Pir, piriform cortex (scale bar, 2.5 mm). (C) Frontal section at the level of the dorsal raphe nucleus (DR). IC, inferior colliculus; SC, superior colliculus; CG, central gray; MnR, median raphe; IP, interpeduncular nucleus (scale bar, 2 mm).

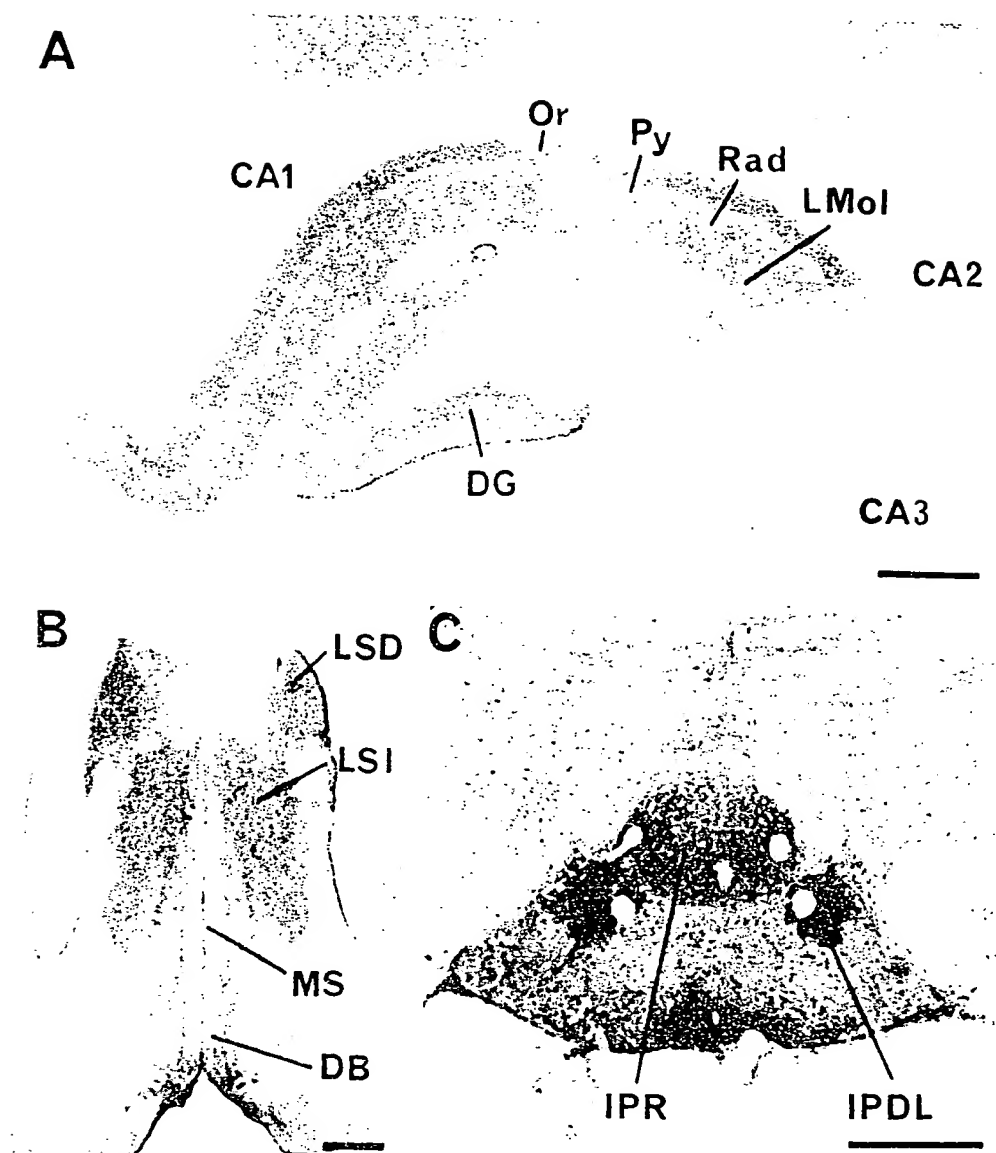


Fig. 10. Brain areas with immunostaining by purified anti-5-HT_{1A}-i3 GST antibodies mainly confined to the neuropile. (A) Dorsal hippocampus with strong immunolabelling of the neuropile in the dentate gyrus (DG), and stratum oriens (Or) and stratum radiatum (Rad) of Ammon's horn. In contrast, no immunoreactivity was observed in the pyramidal cell layer (Py), CA1, CA2, CA3, subfields of Ammon's horn; LMol, stratum lacunosum moleculare (scale bar, 0.5 mm). (B) Septal area with intense immunostaining of the neuropile in the dorsal nucleus and intermediate nucleus of the lateral septum. Abbreviations are as in Fig. 9A (scale bar, 0.5 mm). (C) Heterogeneous immunostaining of the interpeduncular nucleus with the dorsolateral subnucleus (IPDL) showing the most intense immunoreactivity. IPR, rostral subnucleus (scale bar, 0.5 mm).

DISCUSSION

The 5-HT_{1A} receptor belongs to the G-protein-coupled receptor superfamily, whose members are known to possess seven hydrophobic transmembrane domains, linking the intracellular and extracellular loops.² As confirmed herein by comparing the aa sequence of the i3 loop of 11 different 5-HT receptors of this superfamily, this portion of the sequence shows the lowest degree of homology between G-protein-coupled receptors.² We therefore selected this

portion of the aa sequence of the 5-HT_{1A} receptor to make a fusion protein as an alternative to synthetic peptides, to be used as antigen for raising specific anti-5-HT_{1A} receptor antibodies in rabbits. Indeed, the synthetic peptide that we used previously contained only 26 aa, whereas the 5-HT_{1A}-i3-GST fusion protein made in the present study was composed of the complete i3 loop, i.e. 135 aa. Such an antigen could exhibit more epitopes than a synthetic peptide and thus could be more immunogenic. Furthermore, because of their larger size, fusion

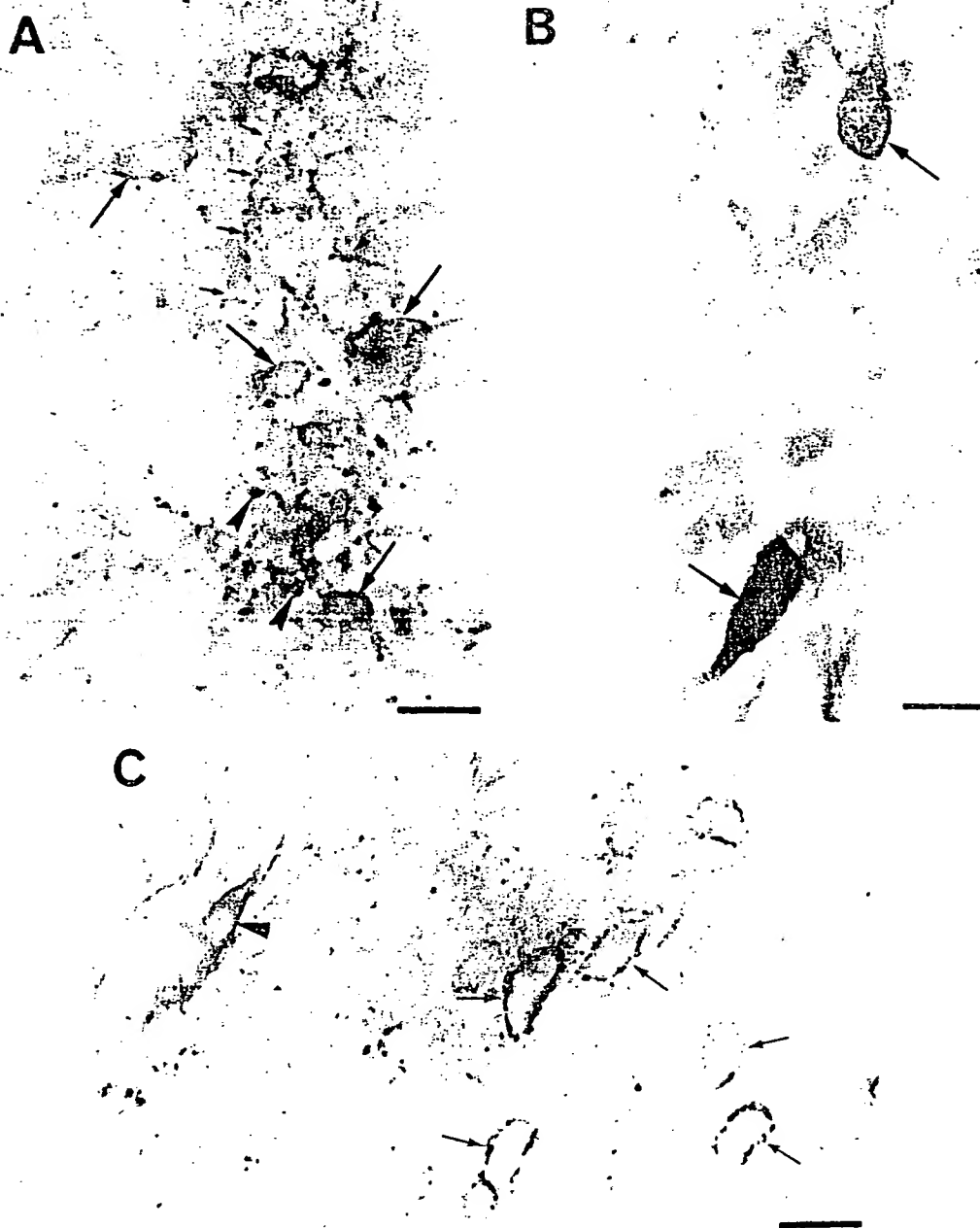


Fig. 11. Perikarya and dendrites immunostained by purified anti-5-HT_{1A}-i3 GST antibodies in three different regions of the rat brain. (A) High magnification (scale bar, 20 μ m) of the median raphe nucleus showing perikarya outlined by plasma membrane immunostaining (large arrows). An immunoreactive dendrite (small arrows) can be followed on \sim 60 μ m, and other immunostained profiles (arrow heads) probably correspond to cross-sections of such dendrites. (B) Two immunostained perikarya in the vertical limb of the diagonal band (scale bar, 20 μ m). (C) Immunostained perikarya in the zona incerta. Immunolabelling is homogeneously distributed in the plasma membrane of some cells (arrow head) or is uneven with a patchy appearance on other cells (arrows; scale bar, 20 μ m).

proteins might adopt a secondary, or ternary conformation, closely related to the native receptor. This approach has already been used successfully to produce antibodies against muscarinic cholinergic receptors,^{31,49,52} dopamine D₁ and D₂ receptors,^{5,30} a metabotropic glutamate receptor⁴² and the 5-HT_{3A}-A receptor.⁴⁶

In the present case, this strategy also appeared to be efficient, since antibodies could already be detected in the rabbit serum one month after the first injection of the 5-HT_{1A}-i3/GST fusion protein using both ELISA and immunoprecipitation of 5-HT_{1A} receptors solubilized from rat hippocampal membranes. In the four rabbits used for the immunization, the responses

were similar, and maximal titers of the antisera were reached after only two injections of the antigen, and then plateaued for the following four months of booster injections.

As expected from the enclosing of the 26 aa synthetic peptide sequence Gly²⁴³-Glu²⁶⁸ in the 5-HT_{1A}-i3 GST fusion protein, antibodies raised against this peptide⁹ bound to this protein antigen, as shown by the positive reaction in the ELISA test. In contrast, anti-5-HT_{1A}-i3 GST antibodies did not recognize the 26 aa synthetic peptide. Several interpretations can be proposed to explain this negative result. Indeed, within the i3 loop, certain portions other than the 26 aa Gly²⁴³-Glu²⁶⁸ sequence might be more immunogenic than the latter, making the rabbits preferentially raise antibodies to epitopes outside this sequence. Alternatively, the Gly²⁴³-Glu²⁶⁸ sequence might be hidden because of the secondary ternary conformation adopted by the 5-HT_{1A}-i3/GST fusion protein, allowing only other epitopes to be available for the production of antibodies. Finally, it cannot be excluded that the fusion protein was rapidly hydrolysed in the rabbit, in a way which cut the Gly²⁴³-Glu²⁶⁸ sequence.

In addition to the aa sequence corresponding to the synthetic peptide previously used in raising anti-5-HT_{1A} receptor antibodies,⁹ N- and C-terminal portions of i3 probably also contained no epitopes recognized by the anti-fusion protein antibodies. Indeed, these portions have been shown to play a critical role in the interaction of receptors with G proteins,² but no change in this interaction could be found here when the 5-HT_{1A} receptors were exposed to the anti-5-HT_{1A}-i3 GST antibodies. Thus, neither the inhibitory effect of GppNHP on the specific binding of [³H]8-OH-DPAT to solubilized and membrane-bound 5-HT_{1A} receptors, nor the Gi-dependent inhibition of hippocampal adenylate cyclase due to the stimulation of 5-HT_{1A} receptors^{28,29} were significantly affected upon exposure to anti-5-HT_{1A}-i3 GST antibodies. That the N- and C-terminal portions of anti-5-HT_{1A}-i3 probably play no role in the immune response is further supported by the fact that anti-5-HT_{1A}-i3 GST antibodies were able to immunoprecipitate 5-HT_{1A} receptors but not 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors (and also 5-HT₃, α_1 - β -adrenergic receptors) solubilized from rat hippocampal membranes, in spite of a relatively high degree of sequence homology at these particular portions (see Fig. 1B). Indeed, both the N- and C-terminal parts of i3 of the 5-HT_{1A} receptor are rather hydrophobic, and may therefore be hidden in the ternary conformation adopted by the fusion protein, preventing their identification as potential epitopes for the production of antibodies in rabbits. Accordingly, the epitopes recognized by the anti-5-HT_{1A}-i3 GST antibodies should be located within the intermediate parts (aa 225-240 and/or aa 275-335) of i3, but further investigations with synthetic peptides corresponding to these sequences are

necessary to really demonstrate this inference.

Clearly, anti-GST antibodies were also present in the anti-5-HT_{1A}-i3/GST antisera, as shown in Western blotting experiments with pure GST. However, anti-GST antibodies could be extensively removed by passing anti-5-HT_{1A}-i3/GST antisera through a GST-Affigel 15 affinity column. In addition, anti-GST antibodies did not interfere with the recognition of 5-HT_{1A} receptors by these antisera. Thus, purified anti-GST antibodies gave no immunautoradiographic labelling in sections of the rat brain and saturation of anti-5-HT_{1A}-i3/GST antibodies by pure GST did not affect the immunolabelling by anti-5-HT_{1A}-i3/GST antiserum.

Immunautoradiographic labelling by anti-5-HT_{1A}-i3/GST antibodies superimposed perfectly with the autoradiographic labelling of 5-HT_{1A} receptors by specific 5-HT_{1A} receptor radioligands such as [³H]8-OH-DPAT,²⁷ [¹²⁵I]BH-8-MeO-NPAT¹⁴ or [³H]5-methyl-urapidil.²⁹ In particular, intense labelling was found in the limbic areas (septum, hippocampus, entorhinal cortex) and the dorsal raphe nucleus, whereas no labelling could be detected in the striatum, substantia nigra and cerebellum, where 5-HT_{1A} receptors are either absent or at a very low density.²⁷ In contrast, the latter structures are enriched in 5-HT_{1B} receptors. The lack of immunoreaction at their level further confirmed data obtained in immunoprecipitation experiments where no cross-reactivity was found between anti-5-HT_{1A}-i3 GST antibodies and 5-HT_{1B} receptors (in spite of some sequence homology in the N- and C-terminal portions of i3 in 5-HT_{1A} and 5-HT_{1B} receptors: see Fig. 1B).

One of the main goals of the production of specific antibodies is the immunostaining of the corresponding antigen at the cellular and subcellular levels using light and electron microscopy. Clearly, anti-5-HT_{1A}-i3 GST antibodies are useful tools for reaching these goals, as the corresponding immunoreactive material could be visualized at the cellular level using the classical avidin-biotin-peroxidase technique. Interestingly, mainly the neuropile was labelled in the septum, hippocampus and interpeduncular nucleus, whereas both the somas and dendrites were labelled in anterior raphe nuclei. Such differential locations suggest that the addressing of the 5-HT_{1A} receptors may vary to some extent from one cell type (for instance the pyramidal cells in the hippocampus) to another (i.e. the serotonergic neurons in the anterior raphe nuclei⁴³). Immunocytochemical investigations at the electron microscope level are in progress to further explore these differences.

Both the regional and cellular immunostaining by anti-5-HT_{1A}-i3 GST antibodies corresponded exactly to those obtained previously with antibodies raised against the synthetic peptide Gly²⁴³-Glu²⁶⁸.^{9,19,32,39,43} In particular, at the cellular level in the median raphe nucleus, these antibodies to different epitopes (see above) recognized material which was mainly

confined to the plasma membrane of somas and dendrites, as expected of the 5-HT_{1A} (auto)receptor.⁴³ Similarly, in cell cultures, only the plasma membrane of neurons from the mesencephalon, hippocampus and septum of fetal rats could be immunostained by both the anti-peptide antibodies (Ref. 19, and Riad *et al.*, in preparation) and the anti-5-HT_{1A}-i3 GST antibodies (unpublished observations).

CONCLUSION

To date, antibodies to various 5-HT receptors have been made: anti-5-HT_{1A} (see Refs 4, 9, 12, 38, 51 and this paper) anti-5-HT_{1B},²³ anti-5-HT_{2A},^{13,35} anti-5-HT_{2C},⁴⁶ and anti-idiotypic antibodies to 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors.⁴⁴ Although the specificity of these antibodies might have not been

thoroughly proven for all of them (see Introduction), they generally constitute excellent tools to investigate important questions such as the differential location of these receptors at the subcellular level and their possible co-expression within only one cell, as suggested by electrophysiological observations.³

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